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Rapid diagnostic tests for malaria: A review

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Abstract

Malaria remains one of the most important endemic disease threats facing the Canadian Forces during overseas deployment. The existing, accepted gold-standard for diagnosing malaria is the microscopic examination of thick and thin blood smears. This method has the advantage of high sensitivity, quantifiable results, and accurate speciation, but is fairly time-consuming and requires well-trained microscopists in order to detect low parasitemias and to properly differentiate the species. Commercially available rapid diagnostic immunocapture test strips now exist which do not require the same level of training and equipment as microscopic examination, and are also significantly faster. However, as this review delineates, clinical trials have shown that the strips are not as sensitive as microscopic examination in detecting low level parasitemias, cannot quantify the level of malaria infection, and, at present, can only differentiate between falciparum and non-falciparum malaria. The strips also have problems relating to antigen persistence in the blood after parasite clearance from chemotherapy, leading to false positive post-therapeutic diagnoses. At present, the test strips are not approved by Health Canada and any use must be under appropriate clincial trial conditions. In addition, the test strips are currently not recommended to be used without a parallel blood smear sample being examined.

Résumé

La malaria demeure l'une des menaces les plus importantes de maladies endémiques à laquelle doivent faire face les Forces canadiennes durant leur déploiement à l'étranger. Les normes idéales actuellement reconnues pour diagnostiquer la malaria consiste en un examen microscopique de frottis de sang épais ou fluide. La méthode a l'avantage de produire des résultats quantifiables de grande sensibilité et de différencier les espèces avec précision mais cette méthode absorbe du temps et requiert des microscopistes expérimentés capables de détecter les parasitémies faibles et capables de différencier correctement les espèces. Des bâtonnets diagnostiques d'immunocapture de diagnostic rapide offerts sur le marché sont actuellement disponibles mais ne requièrent pas le même niveau d'expérience et d'équipement que les examens microscopiques et ils sont aussi beaucoup plus rapides. Cette étude a cependant délinéé que les essais cliniques ne sont pas aussi sensibles que les examens microscopiques pour détecter les parasitémies de bas niveau, qu'ils ne quantifient pas le niveau de l'infection de la malaria et qu'à présent, ils ne différencient qu'entre la malaria fanciparum et la malaria non-fanciparum. Les bâtonnets ont aussi des problèmes à interpréter la persistance d'antigènes dans le sang après la disparition des parasites causée par la chimiothérapie, ce qui aboutit à des diagnostics post-thérapeutiques faux positifs. Actuellement, les bâtonnets diagnostiques ne sont pas approuvés par Santé Canada et ils ne doivent être utilisés que dans des conditions d'essais cliniques appropriés. De plus, il n'est pas actuellement recommandé d'utiliser les bâtonnets diagnostiques sans effectuer parallèlement l'examen d'un échantillon de frottis sanguin.

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Executive summary

Rapid diagnostic tests for malaria

Bradley J. Berger; DRDC Suffield TM 2005-099; Defence R&D Canada – Suffield; June 2005.

Background

Malaria remains one of the most important local disease threats encountered by the Canadian Forces during a number of their overseas deployment. As a malaria infection can rapidly lead to death, it is very important to be able to quickly and accurately diagnose malaria cases. This paper reviews the published literature relating to rapid diagnostic tests for malaria in response to a request from the Canadian Forces Health Services Group.

Principal results

The current accepted gold-standard for diagnosing malaria cases remains the same as for over 100 years: the microscopic examination of blood smears stained with Giemsa or Field's stain. This technique has the advantage of being very sensitive, being able to quantify the number of parasites present in the blood, and being able to differentiate the four species of human malaria. However, microscopic examination is time-consuming, requires an oil-immersion microscope, and also requires a well-trained individual in order to accurately detect low parasitemias and properly speciate the parasites involved. Commercially available rapid diagnostic immunocapture test strips exist which can differentiate *Plasmodium falciparum* malaria from the other three species of the disease, and which are more rapid and easier than microscopic examination. Unfortunately, the test strips are not as sensitive as microscopy for detecting low level malaria infections (approximately <500 parasites/µl of blood) and cannot quantify the parasite load in the patient. In addition, the test strips suffer from the phenomenon of antigen persistence, where the protein detected by the strips circulates in the blood for days after the parasites have been successfully killed by antimalarial drugs. These post-therapeutic false-positive readings may be improperly interpreted as the presence of drug-resistant malaria. While the test strips are fully portable, they require storage between 4-30^{o}C, which may be impossible for individuals on tropical deployment.

Significance of results

Despite clinical trial in over 20,000 individuals, the test strips remain unapproved by Health Canada. Therefore, any examination of the tests by the Canadian Forces would require a properly approved clinical trial. At present, Health Canada, FDA, and WHO all recommend that rapid diagnostic test strips not be used on their own to diagnose malaria, but must be accompanied by a microscopic examination of a blood smear. The issue of individual patient self-examination using the strips has only been briefly studied, and may bear some future examination in the context of soldiers remote from clinical assistance for extended periods of time.

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Sommaire

Rapid diagnostic tests for malaria

Bradley J. Berger; DRDC Suffield TM 2005-099; R & D pour la défense Canada – Suffield; June 2005.

Situation générale

La malaria demeure l'une des menaces les plus importantes de maladies locales que les Forces canadiennes doivent affronter durant un certain nombre de leur déploiement à l'étranger. L'infection peut rapidement causer la mort et il est très important de pouvoir rapidement et correctement diagnostiquer les cas de malaria. Donnant suite à une demande du Groupe des services de santé des Forces canadiennes, cet article examine la documentation publiée au sujet des tests de diagnostic rapides de la malaria.

Résultats

Les normes idéales qui sont actuellement admises pour diagnostiquer les cas de malaria n'ont pas changé depuis 100 ans. Il s'agit de l'examen microscopique de frottis de sang contaminé par un colorant Giemsa ou Field. Cette technique a l'avantage d'être très sensible; elle permet de quantifier le nombre de parasites présents dans le sang et de différencier quatre espèces de malaria chez les humains. L'examen microscopique absorbe cependant beaucoup de temps; il requiert aussi un microscope à bain d'huile ainsi qu'un individu expérimenté capable de détecter avec précision des parasitémies faibles et de spécifier correctement les parasites présents. Il existe des bâtonnets diagnostiques d'immunocapture de diagnostic rapide offerts sur le marché capables de différencier la malaria *Plasmodium falciparum* de trois autres espèces de la maladie et ces tests sont plus rapides et plus faciles à utiliser que l'examen microscopique. Les bâtonnets diagnostiques ne sont malheureusement pas aussi sensibles que la microscopie pour détecter les infections de faible niveau (<500 parasites/µl de sang environ) et ne sont pas en mesure de quantifier la densité parasitaire chez le patient. De plus, les bâtonnets diagnostiques souffrent du phénomène de la persistance des antigènes ; les antigènes sont encore présents quand la protéine détectée par les bâtonnets circule dans le sang pendant plusieurs jours après que les drogues antipaludiques aient réussi à détruire les parasites Ces résultats post thérapeutiques faux positifs sont parfois mal interprétés comme signifiant la présence de malaria pharmacorésistante. Les bâtonnets diagnostiques sont facilement transportables mais ils doivent être entreposés entre 4 et 30^{o}C ce qui s'avère parfois impossible lors des déploiements dans les pays tropicaux.

Portée

Les essais cliniques ont été effectués sur plus de 20 000 individus mais les bâtonnets diagnostiques n'ont toujours pas été approuvés par Santé Canada. Par conséquent, Les Forces canadiennes ne sont pas en mesure d'utiliser ces tests sans que les essais cliniques soient préalablement sanctionnés. Santé Canada, le FDA et l'OMS recommandent actuellement que les tests rapides par bâtonnets diagnostiques ne soient pas utilisés par eux-mêmes pour diagnostiquer la malaria mais qu'ils soient accompagnés d'un examen microscopique de frottis sanguin. Le problème d'auto-examen par le patient utilisant les bâtonnets diagnostiques n'a été étudié que brièvement et il se peut qu'il soit réexaminé, dans le contexte de soldats éloignés de soins cliniques, durant une durée prolongée.

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Introduction

Malaria remains one of the top sources of mortality and morbidity in the world, with over 200 million infections and over 1 million deaths per year [1]. In overseas deployments of the Canadian Forces (CF), malaria is also one of the main endemic disease threats to troops. As malignant tertian malaria, caused by *Plasmodium falciparum*, can rapidly lead to death, and other species of malaria, in particular *P. vivax*, have dormant stages that lead to relapsing infections, it is essential to obtain rapid and accurate diagnosis of infection. In addition, the world-wide spread of drug resistant strains of malaria necessitate that patients be accurately screened for elimination of the parasites after chemotherapy. In response to a request from CF Health Services Group, this review addresses the current availability and status of rapid diagnostic tests for malaria, and a brief comparison to standard microscopic testing and polymerase chain reaction (PCR) diagnosis. The status of rapid diagnostic testing for malaria up to 2002 was reviewed by Moody [2], but data from individual clinical trials were not included. This document does not represent a training manual on the specifics of properly conducting malaria diagnostic tests, and interested individuals are advised to examine the technical literature that is packaged with a particular diagnostic test or a text on diagnostic laboratory procedures for the suggested protocols.

Diagnostic Technologies

Microscopy

Standard Thick/Thin Smear Examination

To this day, Giemsa, Field's, or Wright's staining of thick and thin smears of blood collected from finger pricks or venipuncture remains the accepted gold standard for the diagnosis, speciation, and quantification of malaria infection [3]. While it is a very old technique, smear examination still has many advantages that more recent technologies have been unable to surpass. The technique also has some less desirable features that have fuelled the development of more recent tests.

The principle disadvantages to smear examination are the time, equipment, and expertise involved. In as much as the required equipment is a light microscope with an oil immersion lens, slides, Giemsa stain, methanol, and water, the relative initial expense is low and the per assay cost is very low. However, even this small amount of laboratory equipment is unsuitable for a field medic, requiring removal of the patient (or his blood sample) from front-line duties. It is possible, particularly in the context of special forces activities, that individuals might be tasked with activities remote from a suitable diagnostic laboratory for long periods of time. The time per assay is generally considered to be 1-2 hours at best, as it takes about 45 minutes to smear, dry, fix, and stain the slides. Diagnosis of no malaria infection, or infections with low parasitemias, require extended examination of the thick smear and should ideally be performed by two technicians in a blinded manner. The length of time for examining each sample is not excessive, but is certainly not a rapid technique except in those cases with high parasitemias easily quantified in a thin smear. The major disadvantage to slide examination, particularly in labs not seeing large numbers of positive and negative samples on a regular basis, is the expertise required to accurately diagnose infections with low parasitemias, and to accurately speciate the *Plasmodium*. As the course of chemotherapy is determined by the malaria species found on the slide, experience in this technique cannot be over-emphasized. In Canada, potential malaria cases are referred to regional laboratories in order to take advantage of, and maintain, expertise in slide examination.

The main advantages to slide examination are that it provides a permanent record of the sample that can be re-examined as often as necessary. Slides taken at different times pre- and post-therapy can therefore give a very accurate measure of the course of infection and the effectiveness of treatment. The method is fully quantitative, allowing an accurate measure of the degree of infection and, thus, the urgency and type of treatment. Lastly, the method allows for speciation of the infection, which will also play a large role in determining the types of antimalarial prescribed. These last two advantages cannot be over-stated, as no other more recent technique has managed to perform them accurately.

Fluorescent Microscopy

Fluorescent dyes which penetrate erythrocytes and bind DNA, such as acridine orange, can be used to increase the sensitivity of microscopic detection of low parasitemias in malaria infections [4]. While such an approach makes it easier to visualise small numbers of parasites in thick smears, the technique requires a fluorescent microscope, which is substantially more expensive than a standard light microscope. In addition, the dye binds any DNA in the blood sample, so technicians must become skilled in discerning malaria from other potential interferences in the sample. Lastly, only the parasite nuclei become stained with this method, leading to difficulties in speciation of the malaria found in the sample.

Polymerase Chain Reaction (PCR)

While PCR detection of malaria cannot be considered a rapid technique, due to the equipment considerations and length of time per run, there are several important features to its use in detecting and confirming malaria cases. The most important consideration is the sensitivity of PCR in detecting plasmodial DNA in blood samples, as this technique can yield positive diagnoses in patients where the parasitemia is completely undetectable in blood smears [5]. This sensitivity also extends to patients showing no clinical symptoms of malaria [6], which can complicate the decision making process for chemotherapy. Positive tests by PCR are known to persist for a short period after successful antimalarial treatment [7], perhaps due to the sensitivity of this assay to residual parasites not visible in blood smears. The major strength of PCR lies in its ability to confirm cases with very low parasitemia visible in the smears, cases where technicians differ on the presence of parasites in a smear, and cases where speciation of the infection is unclear. This latter feature is particularly valuable, as clear speciation of non-falciparum malaria can often be difficult in blood smears with low parasitemia.

Standard PCR is also a non-quantitative technique, so positive PCR results cannot be directly correlated with a severity of infection. Recent developments in real-time, fluorescent PCR suggest that this technique may ultimately become reliably quantitative [8,9]. However, as of the present date, it is still very much an experimental approach.

Rapid Diagnostic Test Kits

Colorimetric Assays

There exists at present a single commercially available colorimetric assay system for detecting malaria in blood samples. The MalStat reagent from Flow Laboratories (Portland, OR, USA) is based on the differential substrate range of *Plasmodium spp*. lactate dehydrogenase and the human enzyme. The parasite enzyme has a much greater ability to substitute the chemical 3-acetylpyridine adenine dinucleotide (APAD) for nicotinamide adenine dinucleotide (NAD) as the cofactor in converting pyruvate to/from lactate [10]. An aliquot of infected blood is lysed by detergent and incubated with APAD, lactate, diaphorase, and nitroblue tetrazolium for the reaction shown in Figure 1. The resulting blue colour can then be quantified at 595 nm using a

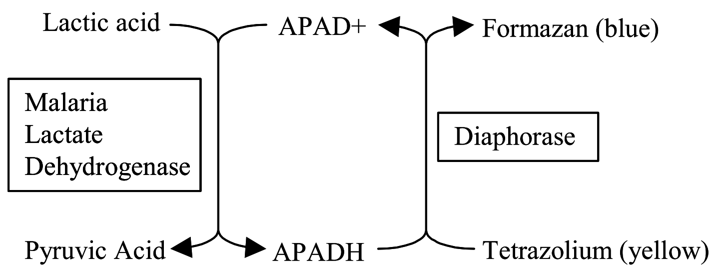

Figure 1: The MalStat reaction for detecting pLDH.

spectrophotometer. The assay is quantitative for the amount of malaria in the blood sample, but is unable to provide any speciation. As a diagnostic tool, this assay requires skilled laboratory personnel, considerable equipment, and fridges/freezers for storing the reagents, and is not suitable for field use. There are no published trials of the use of MalStat reagent alone for the detection of malaria. However, when combined with an anti-*P. falciparum* lactate dehydrogenase antibody, in an immunocapture plate (see Figure 2), MalStat reagent could detect parasitemias as

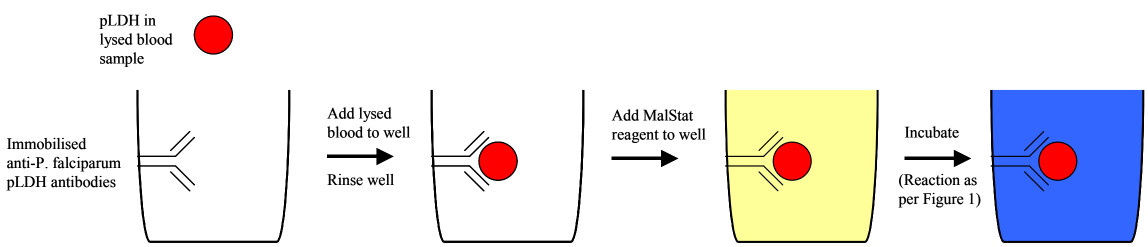

Figure 2: The immunocapture assay for pLDH and the MalStat reagent.

low as 0.01% with 9% false negatives [11]. Parasitemias lower than this value led to a correspondingly higher rate of false negatives. There were no reports of false positives.

The MalStat reagent is unique in that blood samples can be incubated with varying concentrations of antimalarial prior to lysis and addition of the colorimetric reagent in order to accurately assess the drug resistance of the strain [12]. This assay thus provides a non-radioactive alternative to the ^{3}H-hypoxanthine incorporation method that is routinely used to quantify and confirm the presence of drug resistance [13], and MalStat is marketed more towards this particular usage.

Antigen Binding Strips/Cassettes

The field of rapid diagnostic test kits for malaria is almost completely dominated by antigen binding strips or cassettes. These assays work on the same principles as standard pregnancy test kits and consist of lateral diffusion of a solution containing lysed blood and colloidal gold-labelled antibodies down a strip holding another, immobilised antibody (see Figure 3). At

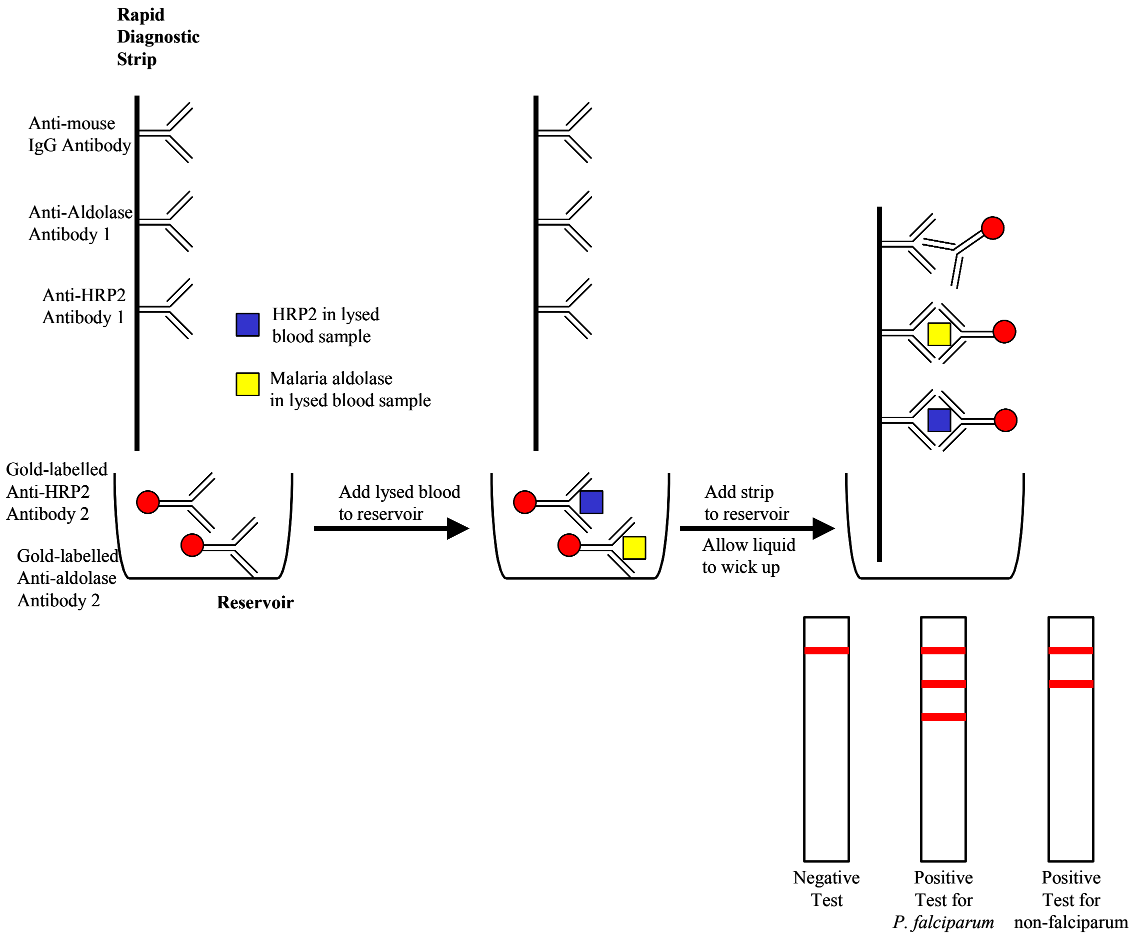

Figure 3: A rapid antigen capture diagnostic test strip for malaria.

present, there are a very large number of suppliers of these malaria test strips (Table 1), which come in two formats: dipsticks, where the strip is placed in a well containing the lysed blood and reagents; and cassettes, where the lysed blood and reagents are added onto the strip in a special holder. Despite the large number of potential suppliers and the very large number of potential malaria antigens, there are actually only two different varieties of test strip: those which detect histidine-rich protein 2 (HRP2) as the *P. falciparum* antigen, and those which detect *P. falciparum* lactate dehydrogenase (pLDH). Detection, of non-falciparum malaria is via a pan-specific antibody, which is generally malarial aldolase or a different epitope on lactate dehydrogenase [2]. All of these test kits are not approved for use in the US or Canada for clinical use, and are for export only (see below for further information on regulatory information). Should anyone prefer to assemble their own HRP2-based *P. falciparum* test kits, the colloidal gold-labelled antibodies and other materials can be obtained from Alchemy Laboratories (Dundee, UK; www.alchemylabs.co.uk/alchemy.html).

Table 1: Currently available malaria rapid diagnostic test strips.

Company	Product	Species	Website
ACON Laboratories	Malaria Pf Rapid Test	Pf*	www.aconlabs.com
All Diag	Palutop+4	Pf Pan	www.alldiag.com
Ameritek	One Step Malaria Test	Pf	www.ameritek.org
Binax	NOW Malaria	Pf Pan	www.binax.com
Bio-Quant	One Step Malaria Test	Pf Pan	www.bio-quant.com
Cellabs	Rapimal	Pf	www.cellabs.co.au
Core Diagnostics	Core Malaria	Pf Pan	www.corediag.com
Cortez Diagnostics	OneStep Rapicard	Pf Pan	www.rapidtest.com
Diamed	OptiMal	Pf Pan	www.diamed.ch
Flow	OptiMal	Pf Pan	www.malariatest.com
Genelabs Diagnostics	Assure Malaria	Pf	www.genelabs.com.sg
Genix Technology**	Malaria Ag	Pf	www.genixtech.com
Global eMed	Malaria Combo Rapid Strip	Pf Pan	www.globalemed.com
Human	Hexagon Malaria	Pf Pan	www.human.de
International Immunodiagnostics	One-Step Malaria	Pf Pan	www.intldiagnostics.com
Kat Medical	KatQuick	Pf	www.katmedical.com
Mega Diagnostics	MegaKwik Malaria	Pf	www.mega-dx.com
Orchid Biomedical	Paracheck Pf	Pf	www.tulipgroup.com
Premier Medical	First Response Malaria	Pf Pan	www.premiermedcorp.com
Princeton BioMeditech	Biosign Malaria	Pf	www.pbmc.com
SPAN Diagnostics	ParaHIT	Pf	www.span.co.in
Standard Diagnostics	Malaria Antigen Test	Pf Pan	www.standardia.com
Trinity Biotech	Rapid UniGold Malaria	Pf	www.trinitybiotech.com
Zephyr Biomedical	Parascreen	Pf Pan	www.tulipgroup.com

*Pf = *Plasmodium falciparum* only; Pf Pan = *P. falciparum* or non-falciparum differentiation

**Canadian company

Histidine-Rich Protein 2

HRP2 is a *Plasmodium falciparum* specific protein which the parasite exports for insertion into the infected erythrocyte membrane at locations known as knobs [14,15]. The protein is also shed into the bloodstream due to the red cell lysis that occurs after parasite schizogony. HRP2 is expressed in all the erythrocytic stages of falciparum malaria except for mature gametocytes, and the protein is not expressed in the early, hepatic stage of the disease. Laboratory strains of *P. falciparum* lacking HRP2 have been characterised [16]. However, it is unclear whether any clinical isolates exist that do not express HRP2.

Lactate Dehydrogenase

pLDH is the terminal glycolytic enzyme in the parasite, and plays a key role in recycling NAD cofactors required for glucose catabolism [17]. This enzyme is found in all erythrocytic stages of the disease, including mature gametocytes, and is also found in all four species of human malaria [18]. Due to differences in the amino acid sequence of pLDH amongst the different malaria species, it has been possible to raise mouse monoclonal antibodies which are either *P. falciparum* specific or *Plasmodium spp.* non-specific [11].

Aldolase

Aldolase is a central glycolytic enzyme that is present in all the erythrocytic stages of the parasite [19,20]. The amino acid sequence of aldolase is virtually identical across a variety of malaria species [21], and antibodies raised against this protein are pan specific [19].

Results of Clinical Trials of Rapid Test Kits

Of the various rapid test strips that have been available, only three have undergone extensive clinical testing for sensitivity and accuracy in comparison to blood smear examination: the OptiMal pLDH test strip (Flow Inc.; Portland, OR, USA), the NOW HRP2 test strip (Binax; Portland, OR, USA), and the ParaSight-F HRP2 test strip (Becton-Dickinson). Unfortunately, despite being the first major malaria rapid test strip and being validated in a large number of clinical diagnostic trials, Becton-Dickinson discontinued the Parasight-F test kit in 2000-2001. The NOW test strip was originally known as the ICT Pf or ICT Pf/Pv, and was originally manufactured in Australia. After passing through several companies, the ICT test was acquired by Binax and renamed NOW.

The OptiMal pLDH test strip was originally available as a *P. falciparum* detection system, but added a second pan-malarial pLDH antibody in order to differentiate falciparum and non-falciparum malarias. This test strip has been tested in over 38 diagnostic trials on several continents, examining both endemic and traveller/immigrant malaria. At present, over 12,500 people have been tested with this strip in published trials with direct comparison to blood smear examination (Table 2). As a general conclusion, the strip performs well in detecting *P. falciparum*, with the test performing poorly on infections with a low parasitemia (generally below approximately 500 parasites/µl blood). For non-falciparum malaria, the test regularly performs poorly, primarily due to the fact that these three species usually present with low parasitemias.

Table 2: Clinical trials of the OptiMal rapid diagnostic test for malaria.

All data is shown relative to microscopic examination of the same sample. Trials are shown chronologically, with the most recently published papers at the top.

Location	**Sample Size**	***P. falciparum***			***P. vivax***			**Ref.**
		False -	**False +**	**Species**	**False -**	**False +**	**Species**	
Brazil	151	0%	0%	100%	0%	0%	100%	[22]
Afghanistan	376	0%	0%	100%	0%	0%	100%	[23]
Nigeria	268	85%*	1.6%	-	-	-	-	[24]
Pakistan	215	5.4%	0%	100%	4.92%	0%	100%	[25]
France**	557	15.5%	1.3%***	100%	14.3%	1.3%***	100%	[26]
Kuwait**	240	0%	-	75.4%	-	-	-	[27]
Peru	72	-	-	-	7.7%	0%	100%	[28]
Canada**	224	38.1%	3.1%	100%	-	-	-	[29]
India	80	4.2%	0%	100%	0%	0%	100%	[30]
Pakistan	499	7.4%	0%	96.3%	11.0%	2.2%	100%	[31]
Laos	894	11%	2.3%	94%	-	-	-	[32]
USA**	216	3.1%	0.4%	100%	0%	0%	100%	[33]
Pakistan	930	18.7%	1.0%	-	31.6%	1.8%	-	[34]

Location	Sample Size	*P. falciparum*			*P. vivax*			Ref.
		False -	**False +**	**Species**	**False -**	**False +**	**Species**	
India	80	4.2%	0%	100%	0%	1.7%	98.2%	[35]
Germany**	539	23.8%	0.3%***	-	12%	0.3%***	-	[36]
Columbia	189	33-60%*	2%	-	3%	2%	-	[37]
Thailand#	1137	62.9%	25.6%	23.1%	82.7%	0.2%	5.7%	[38]
Kuwait**	750	12.8%*	0.6%	-	14.7%*	2.9%	-	[39]
Australia**	158	26.3%	1.6%	100%	20%	0%	96%	[40]
Columbia	112	10.7%	0%	89.3%	1.8%	4.0%	100%	[41]
Vietnam	412	50.3%	0%	-	26.3%*	0%	-	[42]
Myanmar	229	53.2%	1.0%	95.7%	44.0%	2.0%	91.2%	[43]
Malawi	171	60.5%*	7.4%	-	-	-	-	[44]
Laos	97	9.4%	18.2%	100%	0%	0%	100%	[45]
Turkey	190	-	-	-	6.2%	0%	100%	[46]
Tanzania	390	6.0%	0%	100%	-	-	-	[47]
Thailand	175	8.0%	0%	100%	2.4%	0%	100%	[48]
Kuwait**	515	11.1%	0.4%***	-	11.5%	0.4%***	-	[49]
Korea	87	-	-	-	30.8%*	0%	95.6%	[50]
Indonesia	505	28.3%	-	31.4%	51.06%	-	95.5%	[51]
Italy**	139	17%*	-	-	-	-	-	[52]
Germany**	231	11.5%	0.6%	-	-	-	-	[53]
UK**	636	5.0%	0%	100%	95.5%	0%	100%	[54]
UK**	17	5.9%	0%	100%	-	-	-	[55]
Kuwait**	550	30.6%*	0.5%***	-	16.7%	0.5%***	-	[56]
Gambia	401	8.7%	8.0%	95.3%	-	-	-	[57]
UK***/ Columbia	84	23.5%*	0%	100%	0%	0%	100%	[11]
Honduras	202	11.8%*	1.1%	100%	0%	3.9%	100%	[58]

*False negatives predominantly due to low parasitemias

**Travellers, immigrants, and/or soldiers returning from malarious areas

***False positives not broken down by species

#Test storage temperature exceeded specifications

The NOW (ICT) HRP2-based test strips have been equally as well tested in diverse locations. As with OptiMal, NOW/ICT was originally available only for detecting *P. falciparum* malaria, but added a second pan-malarial antibody (in this case against aldolase) for differentiating falciparum and non-falciparum cases. To date, the NOW/ICT test strip has been tested on over 14,000 patients in 38 published clinical trials. For detection of *P. falciparum*, the NOW/ICT strip generally performed slightly better than the OptiMal strip, but suffered from the same problem of a significantly higher rate of false negatives when faced with low parasitemias. For detection of non-falciparum malaria, the strip often performed very poorly and could be considered worse than the OptiMal strip in this regard.

Table 3: Clinical trials of the NOW/ICT rapid diagnostic test for malaria.

All data is shown relative to microscopic examination of the same sample. Trials are shown chronologically, with the most recently published papers at the top.

Location	**Sample Size**	***P. falciparum***			***P. vivax***			**Ref.**
		False -	**False +**	**Species**	**False -**	**False +**	**Species**	
USA**	32	0%	0%	100%	-	-	-	[59]
France**	115	0%	-	100%	0%	-	100%	[60]
France**	557	2.4%	1.1%***	100%	0%	1.1%***	100%	[26]
Germany**	674	0%	0%	100%	62.5%	0.3%	100%	[61]
Kuwait**	240	10.0%	-	90.0%	-	-	-	[27]
Sri Lanka	328	0%	0%	-	30%	-	-	[62]
Canada**	221	25.0%*	0%	100%	-	-	-	[29]
Germany**	2343	0%	0.3%	100%	-	-	-	[63]
Sri Lanka	328	-	-	-	29.3%	0.9%	100%	[64]
Canada**	256	4.0%*	4.3%***	100%	15.3%*	4.3%***	100%	[65]
Thailand	246	0%	3.6%	100%	7.9%	0%	95.2%	[66]
Kuwait**	515	18%	1.0%	-	-	-	-	[67]
Brazil	170	0%	0%	100%	38.2%	0%	100%	[68]
Germany**	495	5.9%	1.5%	96.3%	-	-	-	[36]
Kuwait**	750	11.1%*	1%	-	36.9%*	2%	-	[39]
Australia**	158	2.6%	3.2%	100%	44.0%	0%	100%	[40]
Thailand	559	64.2%*	0.2%	100%	96.9%*	0.1%	98.9%	[69]
India	571	6.0%	6.9%	96.9%	31.5%*	0.5%	98.0%	[70]
Italy**	241	5.6%	5.5%	-	35.7%	0.5%	-	[71]
Vietnam	312	17.4%*	0%	-	80.0%*	0%	-	[42]
Myanmar	229	13.8%*	18.0%	100%	97.1%	0%	100%	[43]
Myanmar	1000	10.3%	6.2%	-	25.6%	5.1%	-	[72]
Philippines	463	5.8%	24.4%	98.3%	27.8%	3.6%	79.7%	[73]
Tanzania	388	0%	26.0%	-	-	-	-	[47]
Kuwait**	515	15.9%	0.9%	98.4%	-	-	-	[49]
Korea	87	-	-	-	55.4%*	0%	100%	[50]
India	344	2.6%	12.4%	100%	27.7%	2.2%	89.4%	[74]
Germany**	231	7.5%	1.7%	-	-	-	-	[53]
Thailand	309	10.4%	4.3%	-	-	-	-	[75]
Cameroon	181	10.9%	5.1%	-	-	-	-	[76]
Indonesia	560	3.0%	7.1%	98.3%	25.0%	8.3%	93.8%	[77]
Singapore **	52	0%	-	94.1%	-	-	-	[78]
Belgium**	251	7.5%	4.1%	99.2%	-	-	-	[79]

Location	Sample Size	*P. falciparum*			*P. vivax*			Ref.
		False -	**False +**	**Species**	**False -**	**False +**	**Species**	
Canada**	200	10.0%	3.8%	93.8%	-	-	-	[80]
Senegal	66	11%	0%	-	-	-	-	[81]
Thailand	305	7.3%	4.9%	94.7%	-	-	-	[82]
France**	156	4%	2%	-	-	-	-	[83]
Honduras	17	35.3%	-	-	-	-	-	[58]

*False negatives predominantly due to low parasitemias

**Travellers, immigrants, and/or soldiers returning from malarious areas

***False positives not broken down by species

In comparing the OptiMal and NOW/ICT test strips, there is no significantly large difference in false positives or false negatives when detecting *P. falciparum*. However, both strips performed poorly when parasitemias were approaching the lower limit of microscopic resolution. Accurate diagnosis of non-falciparum malaria was poor for both strips, with the NOW/ICT strips frequently yielding over 30% false negatives. Both strips appear excellent for accurately distinguishing between falciparum and non-falciparum malaria when the parasitemias are high enough to ensure detection by the strip. It should be pointed out that very few of the published clinical trials had patients with *P. malariae* or *P. ovale* malaria, and these results are not shown in Tables 2 and 3. In general, both OptiMal and NOW/ICT appeared to be very poor at detecting these two species [22,60,61,65].

Of the remaining available test strips, there have been a few published clinical trials which all involve strips detecting HRP2. Their performance has been very similar to that seen for detecting *P. falciparum* with the NOW/ICT strip.

Table 4: Clinical trials of other rapid diagnostic tests for malaria.

All data is shown relative to microscopic examination of the same sample. Trials are shown chronologically, with the most recently published papers at the top.

Location	**Sample Size**	***P. falciparum***			***P. vivax***			**Ref.**
		False -	**False +**	**Species**	**False -**	**False +**	**Species**	
Paracheck								
India	137	0%	8.6%	-	-	-	-	[84]
India	31	0%	-	-	-	-	-	[85]
India	200	5.6%	11.8%	100%	-	-	-	[86]
India	573	5.7%	9.7%	96.7%	-	-	-	[87]
Uganda	742	3%	12%	-	-	-	-	[88]
Vietnam	312	4.2%	0%	-	-	-	-	[42]
Thailand	246	5.7%	2.6%	94.3%	-	-	-	[89]
Kat								
Thailand	90	4%	8%	94%	-	-	-	[90]
Vietnam/Tadjik-istan/Russia**	98	0%	0%	100%	-	-	-	[91]
MakroMed								
Burkina Faso	690	4%	35%	-	-	-	-	[92]
Canada**	200	1.1%	8%	100%	-	-	-	[93]
Malar-Check								
Brazil	65	2.6%	11.5%	-	-	-	-	[94]

**Travellers, immigrants, and/or soldiers returning from malarious areas

Advantages and Disadvantages of Rapid Test Kits

The major advantages to the use of rapid diagnostic strips are the speed of analysis and the ease of use. Once the blood has been collected by finger prick or venipuncture, the assay can be performed in 5-15 minutes, depending on how long it takes the fluid to wick up the strip. The procedure is very simple, and involves adding lysis buffer to the blood sample and application to the strip. In a self-testing trial using the ICT-Pf strip, 153 untrained patients who presented at the Hospital for Tropical Diseases in London for malaria testing were asked to perform a self test using the rapid diagnostic strip [95]. Of these 75% stated that they found the test easy to perform, 84% easy to read the result, and only 9% performed the test incorrectly. Trained laboratory technicians do not appear to have any difficulty performing and interpreting the assay. In terms of speed and ease of use, the rapid test strips are clearly the best alternative of the established and experimental malaria diagnostic procedures.

Unfortunately, the test strips have some less ideal features, especially when compared to blood smear examination. The test strips are qualitative in nature, and thus give no concrete measure of the level of parasitemia. There have been attempts to correlate intensity of the positive band on the strip to parasitemia [66,77], but this approach is very subjective and provides limited information on parasite density. This lack of quantitation is a serious weakness on the part of the rapid test strip, as physicians are unable to determine if the patient needs aggressive antimalarial therapy. In addition, the rapid test strip does not represent as permanent a record of the patient as a blood smear, and the strip cannot be re-assayed by multiple people in a blinded fashion.

The rapid test strips are unable to differentiate between *P. vivax*, *P. ovale*, and *P. malariae*, which also might lead to difficulties in determining the course of treatment. However, the latter two malarias are quite rare, and the most important diagnostic concern is to differentiate falciparum malaria (which is frequently and rapidly fatal) from non-falciparum malaria (which is usually not fatal). While the strips appear to be quite accurate at differentiating falciparum and non-falciparum malaria, the potential for incorrect speciation (0-10% depending on the study) necessitates a parallel blood smear.

Test strip storage remains a potential problem, as storage temperature should not be more than 30^{o}C. In the context of a clinical laboratory facility or CF field medical station, this temperature limitation should not be a problem. However, forward medical personnel will often be at temperatures exceeding 30^{o}C and will thus be prevented from carrying the test strips for on-the-spot diagnosis. This limitation clearly obviates one of the key features of rapid test strips for malaria: the potential for immediate, portable diagnosis.

There have also been reports of false positives, particularly when using certain HRP2 test strips, due to the presence of rheumatoid factor in the blood sample [96-98]. In a comparative study explicitly examining this phenomenon, 26% of samples known to contain rheumatoid factor yielded a positive test for malaria when using the NOW/ICT strip, and 3% when using OptiMal [98]. Absorbing out the rheumatoid factor prevented the false positive results.

Antigen Persistence

One of the inherent complications in using the current rapid test strips for malaria is the phenomenon of antigen persistence. HRP2 is a particularly stable protein in the human bloodstream and resists degradation for days after release from an infected erythrocyte. In several studies, HRP2-dependent test strips (such as NOW/ICT or Parasight-F) were found to yield false positive *P. falciparum* results up to 28-42 days after parasites were cleared by antimalarial treatment and no longer visible in blood smears [36,99]. Given the world-wide presence of drug-resistant strains of *P. falciparum*, it is possible that false positive test strip results after antimalarial treatment would lead to an erroneous diagnosis of drug-resistant malaria. The patient could then be unnecessarily treated with additional drugs, and an unnecessary alteration in prophylactic regimens recommended for others.

Test strips which detect pLDH or aldolase appear to suffer less from the problem of antigen persistence. Studies found that pLDH positivity roughly paralleled the decline in parasitemia visible by microscopic examination [36,54]. Figure 4 shows a comparison of the persistence of common test antigens for a one week period following chemotherapy.

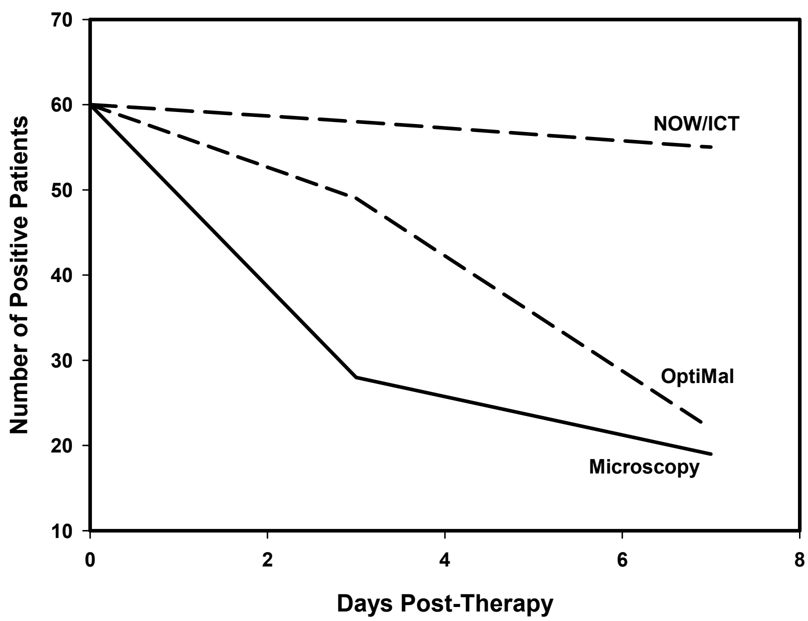

Figure 4: Antigen persistence after antimalarial treatment

The data was adapted from reference [36].

It is unclear whether HRP2 is highly resistant to degradation in the bloodstream, or whether the test is picking up a low level of viable parasites. As the test strips are known to be less sensitive than blood smears, and pLDH positivity does not persist as long, the former possibility seems more likely. Tjitra et al. [100] suggest that HRP2 persistence is due to gametocytemia following clearance of the other blood stages of the disease, but Iqbal et al. [27] have found no relationship between gametocytes and HRP2 persistence.

Assay Sensitivity at Low Parasitemia

Probably the single most important problem with the current rapid diagnostic test strips is the relative lack of sensitivity when compared to the traditional blood smear. Thick blood smears are generally treated as having a lower detection limit of about 20 parasites/µl of blood with a count of 500 white blood cells [101]. Extended viewing of the slide can increase the sensitivity. Field trials with both OptiMal and NOW/ICT strips clearly show that highly accurate diagnosis requires substantially higher parasite densities. Studies where the sensitivity has been broken down by parasite load are shown in Table 5.

Table 5: Assay sensitivity at different parasitemias.

Test Strip	Parasites/µl	Sensitivity for *P. falciparum* (%)	Sensitivity for *P. vivax* (%)	Ref.
OptiMal	<100 >100	0 43	- -	[24]
OptiMal	<500 500-5000 >5000	50 82 96	- - -	[34]
OptiMal	<500 500-5000 >5000	44 98 84	- - -	[39]
OptiMal	<100 100-1000 1000-10000 >10000	0 60 91 100	33 71 84 100	[40]
OptiMal	<1000 1000-10000 >10000	21 49 100	37 100 100	[42]
OptiMal	<61 61-300 >300	7 12 72	0 33 76	[43]
OptiMal	<500 500-1500 1500-5000 >5000	27 56 100 100	- - - -	[44]
OptiMal	<500 500-1000 1000-2000 2000-10000 >10000	83 86 83 88 100	- - - - -	[45]
OptiMal	<100 >100	98 84	- -	[49]
OptiMal	<100 100-500 500-5000	- - -	70 67 68	[50]

Test Strip	Parasites/µl	Sensitivity for *P. falciparum* (%)	Sensitivity for *P. vivax* (%)	Ref.
	>5000	-	75	
OptiMal	<100 100-200 200-500 500-1000 1000-2000	31 53 62 95 94	17 67 57 50 -	[51]
OptiMal	<5 5-50 50-500 500-5000 >5000	73 72 100 100 100	- - - - -	[54]
OptiMal	<50 50-100 100-200 200-300 >300	33 80 100 100 50	45 68 100 100 88	[56]
OptiMal	<50 50-500 500-1500 >1500	60 81 94 100	- - - -	[11]
OptiMal	<100 100-200 200-500 500-1000 1000-2000 >2000	67 100 86 100 100 98	40 100 - 100 75 100	[58]
NOW/ICT	<100 100-1000 1000-10000 >10000	75 96 97 97	50 55 94 100	[65]
NOW/ICT	<100 100-500 500-1000 1000-5000 >5000	0 60 100 100 100	40 67 100 100 100	[66]
NOW/ICT	<500 500-5000 >5000	23* 75* 92*	23* 75* 92*	[39]
NOW/ICT	<100 100-1000 1000-10000 >10000	50 100 100 100	0 7 52 100	[40]
NOW/ICT	<100 100-500 >500	11 48 100	0 0 67	[69]

Test Strip	Parasites/µl	Sensitivity for *P. falciparum* (%)	Sensitivity for *P. vivax* (%)	Ref.
NOW/ICT	<500 500-1000 1000-5000 >5000	- - - -	30 48 91 100	[70]
NOW/ICT	<1000 1000-10000 >10000	27 88 100	0 38 100	[42]
NOW/ICT	<61 61-300 >300	68 88 96	- - -	[43]
NOW/ICT	<100 >100	76 95	- -	[49]
NOW/ICT	<100 100-500 500-5000 >5000	33 44 50 100	- - - -	[50]
NOW/ICT	<60 60-300 >300	40 67 96	- - -	[75]
NOW/ICT	<50 50-100 100-1000 1000-10000 >10000	50 66 89 93 100	- - - - -	[80]

*Combined value for all *P. falciparum* and *P. vivax* samples.

Unfortunately, malaria infections often present with low parasitemias. *P. falciparum*-infected erythrocytes are known to bind to vascular epithelium and are thus sequestered from freely circulating, whereas the other three malaria species only infect specific, limited subpopulations of erythrocytes which keeps parasite numbers lower [3]. As the data from the clinical trials show, both OptiMal and NOW/ICT are simply not dependable at lower parasitemias for both falciparum and non-falciparum malaria. It should be pointed out that OptiMal is now claiming sensitivity down to 50-100 parasites/µl of blood for a new version of the test kit (see www.diamed.ch). Whether this new kit actually meets these claims is not yet clear and might be worthy of further examination.

Current Regulatory Guidelines

At present, WHO, FDA, and Health Canada all require that blood smears be taken in parallel with any use of a rapid diagnostic test strip, and that blood smear examination remains the gold-standard for malaria diagnostics. The following excerpt from the most recent Health Canada document on malaria [102] illustrates the current situation for malaria testing:

> "7. Malaria Diagnosis
>
> It is imperative that a travel history be obtained from all patients with a history of fever, and that thick and thin blood films for malaria be requested urgently for all individuals who have travelled to or through a malaria-endemic area. *P. falciparum* malaria usually presents within 3 months of last exposure; however, it may be delayed in patients who have taken chemoprophylaxis. In addition, other types of malaria, especially that caused by *P. vivax*, may occur months and occasionally up to 5 years after travel in endemic areas.
>
> The treatment of malaria depends upon the species of parasite and the level of parasitemia; therefore, every effort should be made to determine these parameters on an urgent basis. Since malaria is a reportable disease in all provinces/territories, physicians are required to report all cases to the local public health authority.
>
> Occasionally, a single blood film examination may be falsely negative for malaria parasites. Repeat blood films over 48 hours (e.g., every 12 hours x 3) may be required to exclude the possibility of malaria.
>
> The examination of thick and thin blood films by an experienced microscopist is essential for the diagnosis of malaria. The clinical presentation (history and physical examination) of malaria is often non-specific. When malaria is a consideration, especially when the patient may be at risk of *P. falciparum* infection (whether chloroquine-sensitive or not), the laboratory diagnosis and quantification of the level of parasitemia must be considered a medical emergency and performed as soon as possible (< 24-hour turnaround time).
>
> Not all laboratories are proficient in the diagnosis and speciation of malaria. If appropriate expertise cannot be ensured, then the patient should be treated empirically for chloroquine-resistant falciparum malaria and an immediate referral of the patient or the specimen should be made to a specialized facility. These facilities can be identified through the Canadian Malaria Network Centres, listed in Appendix VI.
>
> **While rapid diagnostic tests (RDTs) that use dipstick techniques for the diagnosis of malaria are currently being evaluated in the research setting, none is currently licensed for use in Canada. These RDTs are based on antigen detection of trophozoites and are targeted primarily at *P. falciparum* infections. Some tests differentiate between infections with other species or between falciparum and non-falciparum infection. They are simple to perform and do not require special equipment. They are rapid to interpret and require minimal training to operate. On the other hand, they may remain positive for up to 2 weeks after microscopic clearance, they are relatively expensive compared with microscopy, and they are not quantitative.**

A WHO working group has reviewed the issues surrounding these test kits and identified further research required and possible scenarios for their use. One such scenario would be for self-treatment by travellers to remote areas. Research to date would suggest that this is not feasible, as interpretation by lay people is inaccurate (D II - evidence-based medicine, see Appendix II). There are no data available on self-diagnosis and self-treatment in the long-term traveller or expatriate population. Without training, there is no reason to believe that the efficacy of these interventions will be any better than that demonstrated in the general travel population. However, given that long-term travellers and expatriates represent a reasonably homogeneous group, training in diagnosis and self-treatment (see Section 6), including the use of rapid diagnostic tests for malaria, may prove to be helpful in this population when access to reliable, formal medical care is inadequate. Self-diagnostic kits that require refrigeration will limit access to this technology in some regions.

Polymerase chain reaction (PCR) techniques are also rapidly emerging as a definitive diagnostic tool and can demonstrate impressive sensitivity and specificity (B I - evidence-based medicine). They are, however, limited to laboratories that have the expertise and equipment to conduct these analyses and are still primarily research tools. They are useful as an adjunct to microscopy to confirm cases with low parasitemia and uncertain species. This is particularly useful in Canada, where the incidence of disease is quite low. The Canadian Malaria Network Centres, identified in Appendix VI, can direct clinicians to sites where this technology is available.

Recommendations

The diagnosis of malaria in a suspected case is a medical emergency and requires accurate laboratory testing within a maximum of 24 hours (A I - evidence-based medicine).

Microscopy of Giemsa stained thick and thin smears is the current gold standard for the laboratory diagnosis of malaria (A I - evidence-based medicine). Wright's stained thick and thin smears are used in some laboratories but may miss parasite details that assist in speciation.

PCR has a role in the confirmation of diagnosis but is not accessible widely in a timely fashion, as of yet.

RDTs are of limited utility in the Canadian setting and should not be used as a primary diagnostic tool (DI - evidence-based medicine). "(excerpt from reference [102], with bold-face added by the present author)

WHO continues to work with manufacturers of rapid test strips in order to identify goals that need to be met in order to fully supplement/replace blood smears (see www.wpro.who.int/rdt). In brief, the test strips need to have an improved sensitivity comparable to blood smears, must have less trouble with antigen persistence, and must be more tolerant of high temperatures.

While the manufacturers of the two most tested diagnostic strips are located in the USA, both OptiMal and NOW/ICT are only FDA approved for export to countries that have approved their clinical use. All other use must be treated as a clinical trial, including institutional approval, human-use approval with informed consent forms, and appropriate analysis of the results. Therefore, use of rapid diagnostic strips by the CF would also likely have to be approached in the context of a trial. Given the very high number of patients that have already been examined in the trials outlined in Table 2 and 3, there is unlikely to be much that can be added by a CF trial other than familiarising medical personnel with the assays.

Conclusions

At present, rapid diagnostic assays for malaria do not appear to be superior to blood smear examination, and are not currently approved for use in Canada. Given the large numbers of clinical trials, this lack of approval is primarily due to the lack of corporate underwriting to drive the regulatory process. Indeed, the market for malaria test strips within Canada is likely to be very small. While currently available test strips do have the advantage of a more rapid turn around and portability, the assay sensitivity, antigen persistence, and storage temperature limitations remain hindrances to these assays replacing the blood smear. As these features improve, there will be an increased advantage to using rapid test strips for malaria diagnosis.

The issue of individual end-user diagnosis using these test strips is currently being debated in the context of tourists who plan on being remote from medical assistance for extended periods of time, and can be trained on using the kit and knowing when to use the kit. Certainly there might be possibilities for designing a trial that permits individual CF members to test themselves with the strips in conjunction with a parallel blood smear and test strip by medical personnel. In such a context, it would be wise to test OptiMal and NOW in parallel in order to properly gauge ease of use by lay-persons. The key limits to self diagnosis relate to the ability of the individual to be properly trained to know when to use the strip, to be able to perform and interpret the assay properly, and to follow an appropriate self-medication protocol.

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List of symbols/abbreviations/acronyms/initialisms

CF	Canadian Forces
HRP2	Histidine-Rich Protein 2
pLDH	Parasite Lactate Dehydrogenase

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Malaria remains one of the most important endemic disease threats facing the Canadian Forces during overseas deployment. The existing, accepted gold-standard for diagnosing malaria is the microscopic examination of thick and thin blood smears. This method has the advantage of high sensitivity, quantifiable results, and accurate speciation, but is fairly time-consuming and requires well-trained microscopists in order to detect low parasitemias and to properly differentiate the species. Commercially available rapid diagnostic immunocapture test strips now exist which do not require the same level of training and equipment as microscopic examination, and are also significantly faster. However, as this review delineates, clinical trials have shown that the strips are not as sensitive as microscopic examination in detecting low level parasitemias, cannot quantify the level of malaria infection, and, at present, can only differentiate between falciparum and non-falciparum malaria. The strips also have problems relating to antigen persistence in the blood after parasite clearance from chemotherapy, leading to false positive post-therapeutic diagnoses. At present, the test strips are not approved by Health Canada and any use must be under appropriate clincial trial conditions. In addition, the test strips are currently not recommended to be used without a parallel blood smear sample being examined.

14. KEYWORDS, DESCRIPTORS or IDENTIFIERS (Technically meaningful terms or short phrases that characterize a document and could be helpful in cataloguing the document. They should be selected so that no security classification is required. Identifiers, such as equipment model designation, trade name, military project code name, geographic location may also be included. If possible keywords should be selected from a published thesaurus, e.g. Thesaurus of Engineering and Scientific Terms (TEST) and that thesaurus identified. If it is not possible to select indexing terms which are Unclassified, the classification of each should be indicated as with the title.)

malaria, rapid diagnostic test